Endogenous Ghrelin Attenuates Pressure Overload–Induced Cardiac Hypertrophy via a Cholinergic Anti-Inflammatory Pathway

Yuanjie Mao, Takeshi Tokudome, Ichiro Kishimoto, Kentaro Otani, Hirohito Nishimura, Osamu Yamaguchi, Kinya Otsu, Mikiya Miyazato, Kenji Kangawa

Abstract—Cardiac hypertrophy, which is commonly caused by hypertension, is a major risk factor for heart failure and sudden death. Endogenous ghrelin has been shown to exert a beneficial effect on cardiac dysfunction and postinfarction remodeling via modulation of the autonomic nervous system. However, ghrelin’s ability to attenuate cardiac hypertrophy and its potential mechanism of action are unknown. In this study, cardiac hypertrophy was induced by transverse aortic constriction in ghrelin knockout mice and their wild-type littersmates. After 12 weeks, the ghrelin knockout mice showed significantly increased cardiac hypertrophy compared with wild-type mice, as evidenced by their significantly greater heart weight/tibial length ratios (9.2±1.9 versus 7.9±0.8 mg/mm), left ventricular anterior wall thickness (1.3±0.2 versus 1.0±0.2 mm), and posterior wall thickness (1.1±0.3 versus 0.9±0.1 mm). Furthermore, compared with wild-type mice, ghrelin knockout mice showed suppression of the cholinergic anti-inflammatory pathway, as indicated by reduced parasympathetic nerve activity and higher plasma interleukin-1β and interleukin-6 levels. The administration of either nicotine or ghrelin activated the cholinergic anti-inflammatory pathway and attenuated cardiac hypertrophy in ghrelin knockout mice. In conclusion, our results show that endogenous ghrelin plays a crucial role in the progression of pressure overload–induced cardiac hypertrophy via a mechanism that involves the activation of the cholinergic anti-inflammatory pathway.

Key Words: cardiomegaly ■ ghrelin ■ inflammation ■ mice, knockout

Cardiac hypertrophy is an adaptive response of the heart to persistent increases in hemodynamic workload, such as those caused by hypertension. Although this process is beneficial in terms of normalizing wall stress and oxygen consumption, sustained left ventricular hypertrophy significantly increases the risk of heart failure and sudden death.1 Inflammatory cytokines play an important role in the progression of cardiac hypertrophy; low cytokine levels may be protective for myocytes, whereas persistently high levels are detrimental.3,4

The efferent arm of the vagus nerve can exert anti-inflammatory effects through the α7-nicotinic acetylcholine receptors that are expressed on central nervous system–specific cells and immune cells, including macrophages and CD4+ T cells.5,6 The binding of acetylcholine to α7-nicotinic acetylcholine receptors attenuates the human macrophage production of inflammatory cytokines, such as interleukin (IL)-6, IL-1β, IL-18, and tumor necrosis factor (TNF)-α.7 Stimulation of the vagus nerve by nicotine, a specific α7-nicotinic acetylcholine receptor agonist, has been shown to reduce IL-6 and TNF-α production in sepsis and to reduce inflammatory cell infiltration in a carrageenan air pouch model.8,9 In addition, nicotine-induced reductions in cytokine concentrations have been shown to effectively reduce the incidence of diabetes mellitus in mice,10 attenuate the severity of ulcerative colitis,11 and relieve cardiac hypertrophy in autoimmune myocarditis.12 These effects are produced through what is referred to as the cholinergic anti-inflammatory pathway.13 In hypertension models of varying causes, a deficit in the cholinergic anti-inflammatory pathway contributes to cardiac hypertrophy, and α7nAChR activation relieves the hypertrophy.14

Ghrelin is a growth hormone–releasing peptide originally isolated from the rat stomach in 1999. This peptide was identified as an endogenous ligand for the growth hormone secretagogue receptor,15 which is expressed in the hypothalamus, pituitary, heart, and blood vessels.16 According to many
reports, ghrelin has a cardiovascular role whereby it prevents malignant arrhythmia, improves heart function, and attenuates cardiac remodeling after myocardial infarction. Ghrelin’s mechanism of action seems to be related to the modulation of the autonomic nervous system. Besides suppressing the activation of cardiac sympathetic nerve activity, ghrelin is also apparently able to activate the efferent arm of the vagus nerve in the heart and gastrointestinal tract.

Although ghrelin can theoretically act on the cholinergic anti-inflammatory pathway and therefore be involved in cardiac hypertrophy, this possibility has yet to be fully investigated. We therefore aimed to determine the effects of endogenous ghrelin on cardiac hypertrophy and to assess the contribution of the cholinergic anti-inflammatory pathway by using a pressure overload–induced cardiac hypertrophy model in ghrelin knockout mice.

**Figure 1.** Transverse aortic constriction (TAC) and cardiac hypertrophy. **A,** Typical ascending aortic and left ventricular pressure curves. **B,** The ascending aortic and left ventricular systolic pressures were higher in TAC animals than in sham-operated (Sham) animals. **C,** Typical gross heart samples from each group. **D,** The heart weight (HW)/tibial length (TL) ratio was higher in the knockout (KO) TAC group (n=9) than in the wild-type (WT) TAC group (n=15) at 2 weeks and 12 weeks postoperatively. In each Sham group, n=9. Data are presented as mean±SD. * indicates a significant difference when compared with the Sham group with the same genotype (P<0.05). # indicates a significant difference when compared with the WT TAC group (P<0.05). Ghre indicates Ghrelin; LV, left ventricle; and Nico, nicotine.
Methods
A detailed description of Methods is available in the online Data Supplement.

Administration of Nicotine, Ghrelin, and Methylatropine
First, (−)-nicotine hydrogen tartrate salt (Sigma-Aldrich Corporation, St. Louis, MO) was dissolved in drinking water at a concentration of 125 mg/L and administered orally (≈16 mg/kg per day) from 3 days before the operation until the end of observation.12

Ghrelin was administered by a subcutaneous bolus injection to the neck (1.2 mg/kg per day, equivalent to ≈400 nmol/kg per day) 30 minutes before the surgical procedure and was continuously administered until the end of observation. The dose of ghrelin was selected to generate blood levels in excess of physiological concentrations for >16 hours after injection in gastrectomized mice.22 In cases of methylatropine bromide (1 mg/kg) administration, it was injected intraperitoneally 5 minutes after the ghrelin injection.23

Statistical Analysis
Heart rate variability and hemodynamics were expressed as mean±SEM; other values were expressed as mean±SD. Statistical analysis was performed using Prism version 4 (GraphPad Software Inc, San Diego, CA). The significance of differences observed between groups of mice was analyzed using a 1-way ANOVA followed by the Tukey post hoc test for multiple comparisons. The minimum probability value that was considered significant was 0.05.

Results
Augmented Cardiac Hypertrophy in Ghrelin Knockout Mice
The baseline characteristics of the knockout and wild-type (WT) genotypes are shown in Table S1 in the online-only Data Supplement. When comparing measurements taken before and 12 weeks after the operation, no significant differences were found between the groups with respect to body weight (BW), tibial length (TL), heart rate, and systolic and diastolic blood pressures. However, postoperative BW in the transverse aortic constriction (TAC) groups did not increase in the same manner as it did in the sham-operated (Sham) groups (a tendency toward increase versus the preoperative BW) or the TAC groups that received ghrelin or nicotine (significant increase versus the preoperative BW).

In the TAC groups, the ascending aortic systolic pressure and the left ventricular systolic pressure were 50 to 60 mm Hg higher than the Sham group values (P<0.05; Figure 1A and 1B). No differences were found among the TAC groups. Because only moderate pressure overload was introduced in these mice, no between-group differences were observed in the hemodynamic study with respect to parameters representing systolic function, such as left ventricular ejection fraction and end-systolic elastance (Table S2). However, 12 weeks after TAC, diastolic function in knockout mice was reduced compared with that of WT mice, as evidenced by the slope of the end-diastolic pressure–volume relationship and the τ-index (Table S2).

To evaluate cardiac hypertrophy, we compared heart weights (HWs) at 2 and 12 weeks postoperatively; TL was used as the adjustment factor because BW and growth tendencies were found to depend on whether a TAC or sham operation was performed. Typical heart samples from each group are shown in Figure 1C. Although HW/TL ratios did not differ between the 2 Sham groups, in the TAC groups, knockout mice showed significantly higher HW/TL ratios than WT mice at both 2 and 12 weeks after TAC (P<0.05; Figure 1D). Meanwhile, no between-group differences were observed in lung weight/TL ratios (data not shown).

In addition, echocardiographic examinations were performed 2 and 12 weeks after the TAC or sham operation (Figure 2). In the Sham groups, knockout mice and WT mice had nearly the same left ventricular anterior wall thickness and posterior wall thickness. In the TAC groups, however, the left ventricular anterior wall thickness and posterior wall thickness were significantly greater in knockout mice than in WT mice 12 weeks after TAC (P<0.05).

Inhibited Cholinergic Anti-Inflammatory Pathway in Ghrelin Knockout Mice
Plasma IL-1β, IL-6, and TNF-α levels were determined at 2 days, 2 weeks, and 12 weeks after the operation (Figure 3).
After TAC, IL-1β and IL-6 levels were significantly higher in knockout mice than in WT mice at every time point (P<0.05); TNF-α could not be detected in any group (data not shown).

Heart rate variability in knockout and WT mice was examined at 2 days, 2 weeks, and 12 weeks postoperatively. These data were then used to separately investigate the cardiac autonomic activity of the sympathetic and parasympathetic nerves in rodents. Specifically, the ratio of low-frequency power/high-frequency power (HF) was used to represent relative cardiac sympathetic nerve activity, whereas HF represented absolute parasympathetic nerve activity.24,25 Although there was no significant difference in low-frequency power/HF and HF between the 2 genotypes after the sham operation, knockout mice had significantly lower parasympathetic nerve activity than WT mice at 2 and 12 weeks after TAC (Figure 4).

Ghrelin or Nicotine Administration Activates the Cholinergic Anti-Inflammatory Pathway and Attenuates Cardiac Hypertrophy in Ghrelin Knockout Mice

Ghrelin administration was shown to significantly reduce IL-6 levels in knockout mice 2 days after TAC, but this effect was blocked by the coinjection of methylatropine bromide (Figure S1A). Nicotine was able to reduce both IL-6 and IL-1β levels in knockout TAC mice at 2 days postoperatively (Figure S1B).

When the duration of nicotine or ghrelin administration was extended to 12 weeks postoperatively, both were found to significantly activate the cholinergic anti-inflammatory pathway (Figure 5A and 5B). Parasympathetic nerve activity (as represented by HF) was activated in the knockout TAC group that received ghrelin or nicotine. Ghrelin or nicotine administration also completely suppressed the increases in IL-1β and IL-6 levels in knockout TAC mice (P<0.05).

Nicotine or ghrelin administration into knockout mice significantly attenuated cardiac hypertrophy as indicated by the HW/TL ratio and left ventricular anterior wall thickness, whereas left ventricular posterior wall thickness was only significantly attenuated in the knockout TAC group that received nicotine (P<0.05; Figure 5C and 5D).

Diastolic heart function in knockout TAC mice was inferior to that of WT TAC mice, as evidenced by the r-index and slope of the end-diastolic pressure–volume relationship. Nicotine or ghrelin administration significantly reduced these 2 parameters in knockout TAC mice (P<0.05; Table S2).

Discussion

By comparing knockout TAC mice with WT TAC mice in a pressure overload–induced cardiac hypertrophy model, we were able to show for the first time that the absence of endogenous ghrelin results in severe cardiac hypertrophy and an obvious deterioration in diastolic heart function. This was accompanied by inhibition of the cholinergic anti-inflammatory pathway, as indicated by decreased parasympathetic nerve activity and increased plasma proinflammatory cytokine IL-6 and IL-1β levels. Activation of the cholinergic anti-inflammatory pathway by the administration of nicotine or ghrelin suppresses the increases in IL-6 and IL-1β levels and dramatically improves cardiac hypertrophy and diastolic heart function in knockout TAC mice.

Figure 3. Plasma cytokine levels. A, Plasma interleukin (IL)-6 levels in wild-type (WT) and knockout (KO) mice after sham-operated (Sham) or transverse aortic constriction (TAC) operations measured at 2 days, 2 weeks, and 12 weeks postoperatively. B, Plasma IL-1β levels in WT and KO mice after Sham or TAC operations measured 2 days, 2 weeks, and 12 weeks postoperatively. Data are presented as mean±SD (n=8). * indicates a significant difference when compared with the Sham group with the same genotype (P<0.05). # indicates a significant difference when compared with the WT TAC group (P<0.05).
The cardioprotective effects of ghrelin have been reported in previous studies. In mice with myocardial infarction, ghrelin administration attenuated a collagen volume fraction increase in the noninfarct region. Knockout mice were also shown to experience excessive post–myocardial infarction remodeling, as indicated by an enlarged chamber, an increase in left ventricular wall thickness and the HW/TL ratio, and a deterioration of systolic heart function. The results of this study are consistent with those of previous studies and also demonstrate the importance of endogenous ghrelin in the progression of cardiac hypertrophy.

The elevated circulating IL-1β and IL-6 levels originate from activated immune and nonimmune cells in the pressure-overloaded hearts. Elevated circulating cytokines play an important role in cardiac hypertrophy in both humans and animals. In a recent study, IL-6 infusion in rats caused cardiac hypertrophy and diastolic dysfunction and produced a myocardial phenotype identical to that of a hypertensive heart. The concomitant overexpression of both IL-6 and the IL-6 receptor in mice induced concentric hypertrophy, whereas the removal of IL-6 improves cardiac hypertrophy induced by angiotensin II infusion. By contrast, pressure-mediated hypertrophy and mechanical stretch can generate a subinflammatory level of IL-1β that constitutively maintains an adaptable compensatory hypertrophy.

The ability of ghrelin to suppress inflammation has been demonstrated in numerous diseases, including sepsis, cerebral ischemia, and renal ischemia and reperfusion injury. Under these conditions, ghrelin was shown to reduce plasma levels of proinflammatory cytokines, including IL-6 and TNF-α through activation of the vagus nerve. Our study showed that the effects of endogenous ghrelin can be substituted for by nicotine administration and that the suppression of IL-6 by exogenous ghrelin was completely blocked by methylatropine coinjection, providing further evidence of the involvement of the cholinergic anti-inflammatory pathway.

Ghrelin modulation of the vagal efferent nerve has been well documented by previous studies. Centrally administered ghrelin stimulates pancreatic exocrine secretion through the vagal efferent nerve. Circulating ghrelin also stimulates pancreatic secretion via a vagal cholinergic efferent pathway after gaining access to the brain stem vagovagal circuitry via the area postrema. Using a microdialysis technique, centrally administered ghrelin was shown to activate the cardiac vagal nerve. In addition, peripheral ghrelin was shown to have a moderate effect on parasympathetic nerve activity as represented by HF in healthy controls. Vagotomized subjects did not respond to ghrelin, suggesting the importance of the vagus nerve to the effects of peripheral ghrelin. In this study, knockout mice consistently showed suppressed parasympathetic nerve activity, as indicated by a low HF after TAC, and the suppressed parasympathetic nerves were activated by exogenous ghrelin.

The inhibition of sympathetic nerve activity may also be partially responsible for the effects of ghrelin on cardiac hypertrophy. Sympathetic nerve activity is important for the progress of cardiac hypertrophy, and ghrelin has been shown to have sympathoinhibiting effects. However, sympathoinhibition cannot fully explain ghrelin’s cardioprotective effects. In our previous study, we found that although treatment with the β-blocker metoprolol could reverse post–myocardial infarction cardiac dysfunction in knockout mice, it failed to decrease the augmented gene expression of collagen I, collagen III, and fibronectin. In this study, although there was
However, other mechanisms, including a direct myocardial action of ghrelin, may also be involved in these cardioprotective effects. Moreover, previous studies using genetically modified mice revealed significant sex differences in cardiac phenotypes. It would therefore be prudent to extend our results to the female sex.

**Perspectives**

The endocrine, nervous, and immune systems are the body’s major adaptive systems and are involved in functionally relevant cross-talk to maintain homeostasis. However, the amount of research on the communication between these systems is insufficient. Our study suggests that endogenous ghrelin, a gut hormone, plays a crucial role in attenuating pressure overload–induced cardiac hypertrophy and diastolic dysfunction. These effects are likely due to the activation of the cholinergic anti-inflammatory pathway. By simultaneously evoking sympathetic inhibition and parasympathetic activation, ghrelin was shown to be effective against cardiovascular diseases.

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**Disclosures**

None.

**References**


**Figure 5.** Ghrelin (Ghre) or nicotine (Nico) administration for 12 weeks. A. Ghre or Nico administration lowered plasma interleukin (IL)-6 and IL-1β levels in knockout (KO) mice. In each group, n=8. B. Ghre or Nico administration augmented parasympathetic nervous activity as represented by high-frequency power (HF). C. Ghre or Nico administration reduced left ventricular aneurysm wall thickness (LVWth) and left ventricular posterior wall thickness (LVPWth) on echocardiography. In the wild-type (WT) transverse aortic constriction (TAC) group, n=15; in the KO TAC+Nico group, n=16; in other groups, n=9. D. Ghre or Nico administration attenuated the heart weight (HW)/tibial length (TL) ratio in the WT TAC group, n=15; in the KO TAC+Nico group, n=16; in other groups, n=9. The data presented here are mean±SD (except in B, where mean±SEM was graphed). * indicates a significant difference when compared with the WT TAC group (P<0.05). # indicates a significant difference when compared with the KO TAC group (P<0.05). LF indicates low-frequency power.
What Is New?

• This is the first study to demonstrate that endogenous ghrelin attenuates cardiac hypertrophy and diabetic dysfunction in hearts subjected to pressure overload through a mechanism that involves the cholinergic anti-inflammatory pathway.

What Is Relevant?

• This study provides further evidence in support of the cholinergic anti-inflammatory pathway as a therapeutic target for endogenous ghrelin deficiency– or inflammatory-related cardiac hypertrophy.

Novelty and Significance

Summary

Endogenous ghrelin plays a crucial role in the progression of pressure overload–induced cardiac hypertrophy, and this likely occurs through the activation of the cholinergic anti-inflammatory pathway.
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ONLINE DATA SUPPLEMENT

ENDOGENOUS GHRELIN ATTENUATES PRESSURE OVERLOAD–INDUCED CARDIAC HYPERTROPHY VIA A CHOLINERGIC ANTI-INFLAMMATORY PATHWAY

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Short title: Endogenous ghrelin attenuates cardiac hypertrophy

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Supplementary Methods

Animal models of transverse aortic constriction (TAC)

All experiments were approved by the Animal Ethics Committee of the National Cerebral and Cardiovascular Center Research Institute and conducted in accordance with the guidelines of the Physiological Society of Japan. Constriction of the transverse thoracic aorta was induced in male ghrelin-knockout (KO) mice and their wild-type (WT) C57BL/6J littermates (12–14 weeks of age). A total of 156 WT mice and 184 KO mice were used in the experiments. All animals received a subcutaneous injection of carprofen analgesia (5 mg/kg) and streptcin antibiotics (0.1 mL/kg) before surgery. They were anesthetized with isoflurane (induced at 4%, maintained at 1.5%), intubated, and supported by an animal ventilator (stroke volume, 150 μL; respiratory rate, 135 strokes/min). After a left thoracotomy, the aorta was visualized and a 6.0 Prolene suture was placed around the aorta distal to the brachiocephalic artery. The suture was tightened around a blunt 26-gauge needle placed adjacent to the aorta. The needle was then removed, the chest was closed, and the mice were allowed to recover. Sham-operated (Sham) mice underwent the same surgical procedure without constriction. All mice were on a 12-h light/12-h dark cycle at 25°C and were provided with food and water ad libitum.

Measurement of conscious arterial blood pressure and heart rate

Heart rate and systolic and diastolic blood pressure were recorded using an automatic sphygmomanometer tail-cuff pressure transducer (BP-98A; Softron Co. Ltd., Tokyo, Japan). Each recorded value was derived from three consecutive measurements, which were then averaged to give a single value that was representative of each experimental condition.

Echocardiography

Echocardiographic studies were performed using an echocardiography system equipped with an 18-MHz phased-array transducer (MS400; VisualSonics Inc., Ontario, Canada) under isoflurane anesthesia 2 and 12 weeks after the experimental TAC or sham operation. Left ventricular end-diastolic diameter (LVDd), LV end-systolic diameter (LVDs), percent fractional shortening (%FS), LV anterior wall thickness (LVAWth), and LV posterior wall thickness (LVPWth) were calculated. These variables were used in the cube-function formula for ASE guidelines to calculate LV mass (ASE-cube formula): LV mass (g) = 0.80[1.04{(LVAWth + LVDd + LVPWth) - LVDd}^3] + 0.6.

Heart rate variability analysis

The mice were anesthetized with intraperitoneal injections of combination urethane-chloralose (750 and 35 mg/kg, respectively) with supplemental doses as needed. Electrocardiography signals were then recorded using a physiological analysis system (Bio Amp; AD Instruments, Mountain View, CA, USA). After the heart rate had stabilized, we recorded baseline values for a minimum of 30 min. Autonomic nervous function was examined using a power spectral analysis of heart rate variability (LabChart Pro 7.0; AD Instruments). Heart rate was used to generate a power spectral density curve using the fast Fourier transform. The range of the low-frequency (LF; 0.4–1.5 Hz) or high-frequency (HF; 1.5–5 Hz) component was chosen based on the findings of previous studies. Using these data,
we calculated the parameters of LF power, HF power, and the ratio of LF to HF power (LF/HF).

**Hemodynamic studies**

After heart rate variability was measured, hemodynamic studies were performed as described previously. After anesthetization and intubation, a 1F Mikro-Tip Ultra-Miniature Pressure-Volume catheter (Millar Instruments, Houston, TX, USA) was inserted into the ascending aorta and left ventricle through the right carotid artery to record baseline hemodynamics in the closed chest using a Pressure-Volume Conductance System (Millar Instruments) connected to a physiological recorder (PowerLab system; AD Instruments). Next, a right thoracotomy was performed, followed by transient occlusion of the thoracic vena cava with a silk thread placed around just above the diaphragm to vary the venous return during recording of the hemodynamics. Subsequently, parallel conductance (Vp) was determined by the injection of 10 μL of 15% saline into the left jugular vein to establish the offset due to the conductivity of the structures external to the blood pool. The derived Vp was used to correct the pressure-volume loop data. All of the data were analyzed with the PVAN 3.4 software package (Millar Instruments).

**Cytokine determination**

The levels of interleukin (IL)-1β, IL-6, and tumor necrosis factor-α in the plasma were measured at 2 days, 2 weeks, and 12 weeks after the operations using commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer’s instructions (R&D Systems, Inc., Minneapolis, MN, USA).

**Measurement of plasma ghrelin levels**

Plasma desacyl-ghrelin levels were measured using a Desacyl-Ghrelin ELISA Kit (SCETI, Tokyo, Japan) according to the manufacturer’s instructions.

**Histological analysis**

Twelve weeks after the sham or TAC operation, the murine hearts were excised, fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned. To calculate the collagen content, the heart sections were incubated with Picrosirius Red solution (0.1% Direct Red 80, [Sigma-Aldrich] in saturated aqueous picric acid). The Sirius Red–stained fibrotic area was quantified using Winroof software (Mitani Corp., Fukui, Japan). To measure the myocyte cross-sectional area, the heart sections were incubated with Alexa Fluor® 594–conjugated wheat germ agglutinin (WGA; Life Technologies Corp., Carlsbad, CA, USA). The outline of the myocytes (100 cells/mouse) was traced using the public domain software ImageJ from NIH Image. All of the digital images were recorded using a BIOREVO BZ-9000 microscope (Keyence, Osaka, Japan). The scale bar indicates 100 μm.

**Real-time polymerase chain reaction (PCR)**

Real-time PCR was performed as described previously. Total RNA was extracted from the murine ventricular tissues. First-strand cDNA was synthesized using SUPERSCRIPT II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) from 2 μg of total RNA. To
quantitatively examine levels of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), β-myosin heavy chain (β-MHC), α-skeletal actin (α-SKA), collagen I, and collagen III, real-time PCR amplification using a Light Cycler system (Roche Applied Science, Indianapolis, IN, USA) was performed according to the manufacturer’s instructions. Known concentrations of linearized plasmids containing mouse ANP, BNP, β-MHC, α-SKA, collagen I, and collagen III cDNA were used to generate standard curves. Gene expression was normalized to 36B4. The following primers were designed using GENETYX ver. 9 (GENETYX Corp., Tokyo, Japan): ANP forward, ACC TGC ACC ACC TGG AGG AG; reverse, CCT TGG CTG TTA TCT TCG GTA CCG; BNP forward, CAG CTC TTG AAG GAC CAA GG and reverse, AGA CCC AGG CAG AGT CAG AA; β-MHC forward, GAG CAT TCT CCT GCT GTT TCC and reverse, GAC ACG ATC TTG GCC TTG AC; α-SKA forward, TGC TAT GTG GCC CTG GAC TT and reverse, AAA CGC TCA TTG CCG ATG GTG; collagen I forward, CGA TGG ATT CCC GTT CGA GT and reverse, ATG ACT GTC TTG CCC CAA GT; and collagen III forward, AAT TCT GCC ACC CCG AAC T and reverse, CTG GCC TGA TCC ATA TAG GCA.

References
### Supplementary Tables

#### Tables S1. Mouse characteristics

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<td>66 ± 8</td>
<td>59 ± 9</td>
<td>70 ± 12</td>
<td>69 ± 8</td>
</tr>
<tr>
<td>12 weeks after operation</td>
<td>58 ± 6</td>
<td>59 ± 6</td>
<td>58 ± 8</td>
<td>64 ± 11</td>
<td>71 ± 9 *</td>
<td>69 ± 13</td>
<td>65 ± 10</td>
</tr>
<tr>
<td><strong>Tibial length, mm</strong></td>
<td>17.5 ± 0.5</td>
<td>17.4 ± 0.5</td>
<td>17.4 ± 0.4</td>
<td>17.3 ± 0.8</td>
<td>17.2 ± 0.4</td>
<td>17.5 ± 0.3</td>
<td>17.5 ± 0.6</td>
</tr>
</tbody>
</table>

Data are mean ± SD. *Significant difference compared to baseline (P < 0.05). No significant difference was found between a group after the operation compared to the sham group with the same genotype or compared to the TAC group with the same genotype. KO: ghrelin-knockout; WT: wild-type; TAC: transverse aortic constriction; Sham: sham-operated; Ghre: ghrelin; Nico: nicotine
### Tables S2. Hemodynamic study at 12 weeks after the operation.

<table>
<thead>
<tr>
<th>Variable</th>
<th>WT Sham (n = 8)</th>
<th>WT TAC (n = 9)</th>
<th>WT TAC + Nico (n = 9)</th>
<th>KO Sham (n = 8)</th>
<th>KO TAC (n = 8)</th>
<th>KO TAC + Nico (n = 13)</th>
<th>KO TAC + Ghre (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parameter</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>End-systolic volume, μL</td>
<td>11.0 ± 0.5</td>
<td>11.5 ± 0.4</td>
<td>10.7 ± 0.5</td>
<td>11.0 ± 0.3</td>
<td>8.7 ± 0.4</td>
<td>11.5 ± 0.3</td>
<td>10.7 ± 0.4</td>
</tr>
<tr>
<td>End-diastolic volume, μL</td>
<td>29.3 ± 4.1</td>
<td>33.7 ± 3.1</td>
<td>27.9 ± 3.6</td>
<td>29.3 ± 3.0</td>
<td>21.9 ± 1.9</td>
<td>24.1 ± 1.0</td>
<td>22.5 ± 1.5</td>
</tr>
<tr>
<td>End-systolic pressure, mmHg</td>
<td>87 ± 4</td>
<td>127 ± 6 *</td>
<td>130 ± 8 *</td>
<td>88 ± 3</td>
<td>129 ± 13 *</td>
<td>124 ± 6 *</td>
<td>129 ± 6 *</td>
</tr>
<tr>
<td>End-diastolic pressure, mmHg</td>
<td>8 ± 2</td>
<td>13 ± 3</td>
<td>11 ± 3</td>
<td>9 ± 2</td>
<td>18 ± 7</td>
<td>8 ± 1</td>
<td>9 ± 3</td>
</tr>
<tr>
<td>Stroke volume, μL</td>
<td>20.3 ± 3.9</td>
<td>25.1 ± 3.0</td>
<td>22.2 ± 3.2</td>
<td>20.8 ± 2.6</td>
<td>15.0 ± 1.6</td>
<td>16.3 ± 1.9</td>
<td>16.3 ± 1.9</td>
</tr>
<tr>
<td>Cardiac output, μL/min</td>
<td>9404 ± 1968</td>
<td>10842 ± 1347</td>
<td>9834 ± 1554</td>
<td>9244 ± 1388</td>
<td>6276 ± 867</td>
<td>8627 ± 1123</td>
<td>8627 ± 1123</td>
</tr>
<tr>
<td>Maximal power, mWatts</td>
<td>9.6 ± 1.4</td>
<td>12.9 ± 1.4</td>
<td>9.9 ± 1.2</td>
<td>9.1 ± 1.1</td>
<td>10.4 ± 1.6</td>
<td>10.8 ± 1.0</td>
<td>10.2 ± 1.4</td>
</tr>
<tr>
<td>Ea, mmHg/μL</td>
<td>5.3 ± 1.0</td>
<td>5.5 ± 1.0</td>
<td>5.4 ± 1.2</td>
<td>4.9 ± 0.8</td>
<td>8.2 ± 1.0</td>
<td>8.1 ± 1.0</td>
<td>7.5 ± 0.4</td>
</tr>
<tr>
<td><strong>Systolic indices</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>63.1 ± 4.4</td>
<td>68.3 ± 2.6</td>
<td>66.5 ± 3.8</td>
<td>65.8 ± 2.9</td>
<td>63.4 ± 2.3</td>
<td>63.7 ± 2.1</td>
<td>67.3 ± 4.2</td>
</tr>
<tr>
<td>Stroke work, μL × mmHg</td>
<td>1444 ± 295</td>
<td>2417 ± 307</td>
<td>1543 ± 334</td>
<td>1449 ± 218</td>
<td>1388 ± 225</td>
<td>1348 ± 167</td>
<td>1423 ± 231</td>
</tr>
<tr>
<td>dP/dt max, mmHg/s</td>
<td>7261 ± 908</td>
<td>7848 ± 558</td>
<td>7434 ± 734</td>
<td>6555 ± 632</td>
<td>7162 ± 1201</td>
<td>8497 ± 482</td>
<td>7543 ± 443</td>
</tr>
<tr>
<td>PAMP, mWatts/μL²</td>
<td>124 ± 19</td>
<td>128 ± 19</td>
<td>126 ± 12</td>
<td>119 ± 19</td>
<td>242 ± 69</td>
<td>207 ± 22</td>
<td>195 ± 53</td>
</tr>
<tr>
<td>Ees, mmHg/μL</td>
<td>5.3 ± 0.5</td>
<td>9.5 ± 1.9 *</td>
<td>6.7 ± 1.4</td>
<td>5.4 ± 1.1</td>
<td>11.0 ± 2.1</td>
<td>8.9 ± 1.0</td>
<td>8.4 ± 1.3</td>
</tr>
<tr>
<td>Emax, mmHg/μL</td>
<td>9.0 ± 1.7</td>
<td>15.5 ± 5.6</td>
<td>12.1 ± 5.3</td>
<td>12.0 ± 2.6</td>
<td>13.4 ± 5.8</td>
<td>16.5 ± 2.6</td>
<td>13.4 ± 4.2</td>
</tr>
<tr>
<td>PRSW, mmHg</td>
<td>59.5 ± 14.0</td>
<td>77.4 ± 18.3</td>
<td>67.3 ± 12.5</td>
<td>50.3 ± 10.9</td>
<td>68.7 ± 19.1</td>
<td>63.3 ± 14.0</td>
<td>62.4 ± 12.1</td>
</tr>
<tr>
<td>dP/dt – EDV, mmHg × s⁻¹ × ml⁻¹</td>
<td>259 ± 63</td>
<td>221 ± 40</td>
<td>245 ± 56</td>
<td>209 ± 37</td>
<td>267 ± 57</td>
<td>329 ± 53</td>
<td>316 ± 34</td>
</tr>
<tr>
<td><strong>Diastolic indices</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dP/dt min, mmHg/s</td>
<td>6535 ± 616</td>
<td>7462 ± 398</td>
<td>7235 ± 534</td>
<td>6110 ± 507</td>
<td>6241 ± 751</td>
<td>8449 ± 570</td>
<td>6332 ± 472</td>
</tr>
<tr>
<td>Tau (W), ms</td>
<td>9.3 ± 1.0</td>
<td>9.3 ± 0.8</td>
<td>9.4 ± 0.7</td>
<td>10.4 ± 0.8</td>
<td>13.7 ± 2.1</td>
<td>7.8 ± 0.4</td>
<td>9.3 ± 0.6</td>
</tr>
<tr>
<td>Tau (G), ms</td>
<td>11.8 ± 0.9</td>
<td>11.4 ± 1.0</td>
<td>11.2 ± 0.5</td>
<td>12.5 ± 1.1</td>
<td>14.9 ± 1.0</td>
<td>10.5 ± 0.6</td>
<td>13.3 ± 0.8</td>
</tr>
<tr>
<td>Slope-EDPVR</td>
<td>0.17 ± 0.04</td>
<td>0.23 ± 0.07</td>
<td>0.22 ± 0.04</td>
<td>0.17 ± 0.03</td>
<td>0.57 ± 0.11</td>
<td>0.18 ± 0.08</td>
<td>0.25 ± 0.04</td>
</tr>
</tbody>
</table>
Data are mean ± SEM. *Significant difference compared to the Sham group with the same genotype \( (P < 0.05) \). †Significant difference compared to the WT TAC \( (P < 0.05) \). ‡Significant difference compared to KO TAC \( (P < 0.05) \).

dP/dt-EDV: \( dP/dt \)-end-diastolic volume; Ea: arterial elastance; Ees: end-systolic elastance; Emax: slope of the end-systolic pressure-volume relationship; Ghre: ghrelin; KO: ghrelin-knockout; Nico: nicotine; PAMP: preload-adjusted maximal power; PRSW: preload recruited stroke work; Sham: sham-operated; slope-EDPVR: slope of the end-diastolic pressure-volume relationship; TAC: transverse aortic constriction; WT: wild-type
Figure S1. Alteration in plasma cytokine levels following 2-day ghrelin or nicotine administration. A. Ghrelin administration reduced plasma IL-6 levels in KO mice and was blocked by the co-injection of methylatropine bromide. *Significant difference compared to the TAC + Vehi group (*P < 0.05). #Significant difference compared to the TAC + Ghre group (*P < 0.05). B. Nicotine administration reduced both plasma IL-6 and IL-1β levels in KO mice. *Significant difference compared to the WT TAC group (*P < 0.05); #Significant difference compared to the KO TAC group (*P < 0.05). Data are shown as mean ± SD (n = 8). Atro: methylatropine; Ghre: ghrelin; IL: interleukin; KO: ghrelin-knockout; Nico: nicotine; Sham: sham-operated; WT: wild-type; TAC: transverse aortic constriction; Vehi: vehicle.
Figure S2. *Sirius Red–stained heart sections 12 weeks after the operation.* Collagen volume fraction was calculated in five random fields (n = 5). Data are presented as mean ± SEM. Scale bar: 100 μm. *Significant difference compared to the Sham group with the same genotype (P < 0.05). #Significant difference compared to the WT TAC group (P < 0.05). KO: ghrelin-knockout; Sham: sham-operated; TAC: transverse aortic constriction; WT: wild-type.
Figure S3. Alexa Fluor® 594–conjugated wheat germ agglutinin–stained heart sections at 12 weeks after the operation. Cardiomyocyte cross-sectional area was measured in 100 cardiomyocytes (n = 5). Data are presented as mean ± SEM. Scale bar: 100 μm. *Significant difference compared to the Sham group with the same genotype (P < 0.05). #Significant difference compared to the WT TAC group (P < 0.05). KO: ghrelin-knockout; Sham: sham-operated; TAC: transverse aortic constriction; WT: wild-type.
Figure S4. Expression levels of hypertrophy- and fibrosis-related genes at 12 weeks after the operation. Quantitative gene examinations of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), α-skeletal actin (α-SKA), β-myosin heavy chain (β-MHC), collagen I, and collagen III were made and normalized to 36B4. In each group, n = 7. Data are presented as mean ± SEM. *Significant difference compared to the Sham group with the same genotype (P < 0.05). #Significant difference compared to the WT TAC group (P < 0.05). KO: ghrelin-knockout; Sham: sham-operated; TAC: transverse aortic constriction; WT: wild-type.
Figure S5. Plasma desacyl-ghrelin levels at 2 days and 2 weeks after the operation. No difference was found in wild-type mice at 2 days or 2 weeks after the operation. In each group, n = 6. Data are presented as mean ± SEM. Sham: sham-operated; TAC: transverse aortic constriction.
Figure S6. *Left ventricle masses at 12 weeks after the operation.* The left ventricle masses were calculated based on the measurements on echocardiography. In the WT Sham group, n = 9; in WT TAC group, n= 15. Data are presented as mean ± SEM. *Significant difference compared to the Sham group (P < 0.05). LV: left ventricle; Sham: sham-operated; TAC: transverse aortic constriction; TL: tibial length; WT: wild-type.